Collagen Biosynthesis by Cultured Chinese Hamster Lung Cells. Cell-Free Synthesis of Procollagen α Chains†

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ABSTRACT: Cell-free extracts from the HT1 clone of cultured Chinese hamster lung cells efficiently promote the incorporation of proline into newly synthesized material, 50% of which is digestible to small peptides by highly purified bacterial collagenase. The synthesis of these products occurs under optimal protein synthesis conditions and is inhibited by puromycin. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the cell-free synthesized material reveals a major collagenase sensitive peak (20% of the total product) at mol wt 165 000 which is reflected by a collagenase sensitive material of similar size in the culture medium. Two additional collagenase digestible species (mol wt 95 000 and 65 000), each having a corresponding secreted product, are generated by the cell-free system. These results are consistent with the concept that procollagen is formed by the association of three individually translated proα chains. The data further constitute the report of a highly active homologous cell-free system capable of proα chain biosynthesis derived from a cultured cell line that is a practical source for proα chain mRNA as well as a unique system for elucidating regulatory mechanisms involved in collagen biosynthesis.

The collagen group of proteins represents a variety of distinct genetic types and comprises the major extracellular structural protein of tissue. Current evidence supports the concept that minimally degraded extracellular procollagen has a mass of 450 000 daltons or greater (Bańkowski & Mitchell, 1973), consisting of three disulfide linked subunits designated as proα chains (Monson et al., 1975). Although one laboratory has reported evidence suggesting that procollagen is initially synthesized as a single polypeptide which is subsequently processed to individual proα chains (Church et al., 1971; Park et al., 1975), the majority of evidence supports a model in which each proα chain is individually synthesized (Davidson et al., 1975; Diaz deLeon et al., 1977; Fessler et al., 1975; Monson et al., 1975; Morris et al., 1975; Murphy et al., 1975; Uitto et al., 1976). One approach to elucidate the nature of the initially synthesized product is to characterize the collagenous material obtained by cell-free protein synthesis. Although several laboratories have reported synthesis of collagenous products using polysomes (Lazarides & Lukens, 1971; Kerwar et al., 1973; Kerwar, 1974; Prichard et al., 1974; Collins & Crystal, 1975; Pawlowski et al., 1975) or mRNA preparations (Benveniste et al., 1973, 1976; Boedtker et al., 1974, 1976; Harwood et al., 1974, 1975; Wang et al., 1975; Lee-Own et al., 1977) derived from a wide variety of mammalian and non-mammalian sources, the precise structural characteristics of the cell-free synthesized collagen precursor have not been reported. This reflects the limitations placed by the tissues or cultured cell lines currently used on the amount of collagen specific polysomes or mRNA available for cell-free synthesis. Moreover, recent evidence in cell-free systems indicates that procollagen synthesis may be controlled at the level of translation (Carpousis et al., 1977; Collins & Crystal, 1975). In order to elucidate the precise chemical nature of initially synthesized procollagen and the regulatory elements that control its biosynthesis, and to provide a suitable cell source for procollagen mRNA isolation, it would be advantageous to have available a rapidly growing, easily manipulatable, cloned cell line that synthesizes procollagen both in culture and in a homologous cell-free system as one of its major products.

The data presented in this report indicate that a significant fraction of the protein synthesized by cell-free extracts derived from the HT1 clone of cultured Chinese hamster lung (CHL)[1] cells is collagen-like in nature. The preparations incorporate proline in a highly efficient manner into newly synthesized material with a significant amount of cell-free synthesized product being digestible with purified bacterial collagenase and with a major portion of this material having a molecular weight of 165 000 as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis. These results constitute the report of a highly efficient homologous cell-free system capable of the biosynthesis of collagen proα chains. Since CHL cells grow rapidly to a high density under a variety of culture conditions, it appears likely that this cell line offers an ideal model system, not only as a source for the isolation of sufficient quantities of mRNA to allow detailed characterization of the initially synthesized material, but also for the dissection of the elements involved in the regulation of procollagen biosynthesis.

Experimental Procedures

Materials. Tissue culture medium and fetal calf serum were purchased from Grand Island Biological Company, and plasticware was obtained from Falcon. Radioactively labeled [^{3}H]leucine (sp act. 61 Ci/mmol) and [^{3}H]proline (sp act. 49 Ci/mmol) were obtained from Schwarz/Mann. Tris (Sigma 7–9), bovine serum albumin, β-mercaptoethanol, and Sephadex G-25 were purchased from Sigma. K_2ATP and NaGTP were obtained from P-L Biochemicals, and creatine phosphokinase (rabbit skeletal muscle) and potassium creatine phosphate were purchased from Calbiochem. Electrophoresis reagents and nitrocellulose filters (HAWP, 2.5 cm) were ob-

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[1] Abbreviations used are: CHL, Chinese hamster lung; S30, supernatant fraction from 30 000*g* centrifugation; $NaDodSO_4$, sodium dodecyl sulfate; Tos-LysCH_2Cl, α-*N*-tosyl-L-lysine chloromethyl ketone; CP, creatine phosphate; CPK, creatine phosphokinase.

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tained from Bio-Rad and Millipore, respectively. Purified bacterial collagenase (CLSPA) was obtained from Worthington Biochemicals. Liquid scintillation reagents (naphthalene, 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]-benzene, and Aquasol) were purchased from New England Nuclear; toluene and dioxane were obtained from Fisher Scientific. All inorganic compounds used were ACS reagent grade. The origin of the Chinese hamster lung (CHL) cell line as well as the clone (HT1) employed in these studies have been previously reported (Haralson & Roufa, 1975; Roufa & Reed, 1975).

Growth of Cells and Preparation of Cell-Free Extracts. CHL cells were grown in monolayer culture, and cell-free extracts prepared essentially as previously described (Haralson & Roufa, 1975). Monolayer cultures of CHL cells were grown in 150-mm plastic dishes in Dulbecco's modified Eagle's minimum medium supplemented with glucose and 10% fetal calf serum under a 10% CO_2 atmosphere at 35 °C. When the cultures were 75–90% confluent (~2×10^7 cells/dish), the medium was removed, the plates were washed with phosphate-buffered saline, and the cells were removed by scraping into cold phosphate-buffered saline. The cells were then collected by centrifugation at 1000*g* for 15 min at 4 °C. The yield of cells was approximately 150 mg of cells per dish.

The cell-free postnuclear extract (S30) was prepared by suspending the collected cells in hypotonic buffer (10 mM Tris-HCl (pH 7.5), 10 mM KCl, and 1.5 mM $Mg(OAc)_2$) and allowing the cells to swell for 30 min at 4 °C. Routinely, 5 g (wet wt) of collected cells was suspended in 20 mL of hypotonic buffer. The swollen cells were disrupted in a cold Dounce homogenizer using 10 strokes of a loose fitting pestle followed by 5 strokes of a tight fitting pestle. The homogenate was adjusted to standard buffer and salt conditions by the addition of 1/9 the volume of 300 mM Tris-HCl (pH 7.5), 1.25 M KCl, 50 mM $Mg(OAc)_2$, and 100 mM β-mercaptoethanol. The homogenate was then subjected to centrifugation at 30 000*g* (19 000 rpm in a Beckman JA-20 rotor) for 20 min to remove nuclei, mitochondria, and cell debris. The supernatant fluid (excluding the upper lipid layer) was withdrawn using a Pasteur pipet, and the material (~20 mL) was dialyzed for 8 h against 2×1 L portions of buffer (30 mM Tris-HCl (pH 7.5), 125 mM KCl, 5 mM $Mg(OAc)_2$, and 10 mM β-mercaptoethanol) containing 20% (v/v) glycerol. The dialyzed material was then quick-frozen in aliquots and stored at −70 °C.

Reaction Conditions for Cell-Free Protein Synthesis Directed by CHL S30 Preparations. Standard reaction mixtures contained in a total volume of 0.05 mL: 45 mM Tris-HCl (pH 7.5), 113 mM KCl, 3 mM $Mg(OAc)_2$, 10 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 8 mM creatine phosphate, 2 μg of creatine phosphokinase, 2.04 μM [^{3}H]proline (37 900 cpm/pmol), or 2.05 μM [^{3}H]leucine (48 200 cpm/pmol), and dialyzed S30 extract in the amount indicated. After incubation at 25 °C for the time indicated, the reactions were terminated by the addition of 0.1 mL of 1 N KOH and incubated an additional 20 min at 37 °C to hydrolyze ^{3}H-labeled aminoacyl-tRNA. Trichloroacetic acid (2 mL of a 10% (w/v) solution) was then added to each reaction, and the suspension was placed on ice for 15 min. Acid-insoluble material was collected by filtration through nitrocellulose filters; each filter was washed with 3×4 mL aliquots of 5% trichloroacetic acid followed by 2×4 mL aliquots of deionized water. The filters were then dissolved in a toluene–dioxane based scintillation cocktail (Chiu & Sung, 1970), and radioactivity retained by the filter was determined in a Beckman liquid scintillation counter. Under these conditions, efficiency of counting of each isotope was approximately 36%.

Conditions for Collagenase Digestion of Cell-Free Synthesized Proteins. Standard protein synthesis reactions containing [^{3}H]proline were prepared and incubated as described. The mixtures were then adjusted to 2 mM in respect to $CaCl_2$ and 10 μg of collagenase treated with Tos-LysCH_2Cl to inactivate clostripain (Porter et al., 1971) and assayed to ensure no detectable proteolytic contamination was added. The mixture was then incubated for 90 min at 37 °C, and collagenase digestion of [^{3}H]proline containing proteins was measured by the decrease in acid-insoluble base-resistant radiolabeled material retained by the filter.

Incorporation of [^{3}H]Proline into Secreted Proteins. Cultures of CHL cells (~80% confluent) were washed with Dulbecco's modified Eagle's essential medium supplemented with 2.5% fetal calf serum and were then incubated for 20 h in this medium containing 5×10^{-5} M [^{3}H]proline. The reduced serum conditions were employed to minimize processing of the procollagen (Bańkowski & Mitchell, 1973); it has not been possible, to date, to completely eliminate serum from the growth medium, as CHL cells become nonviable within a few hours under serum-free conditions. After incubation, the medium was collected, and [^{3}H]proline-labeled collagenous proteins were extracted by absorption and elution from glass beads with 1 M NaSCN (Gerard and Mitchell, manuscript in preparation). The eluent was combined and dialyzed against 10 mM Tris-HCl (pH 7.5) and was analyzed as described below.

NaDodSO_4–Polyacrylamide Gel Electrophoresis. Molecular weight analysis of in vitro and in vivo synthesized [^{3}H]-proline-labeled products was performed using 5.0% NaDodSO_4–polyacrylamide gels run under denaturing conditions (Fairbanks et al., 1971). Cell-free synthesized [^{3}H]proline-labeled products (with or without collagenase digestion) were prepared for electrophoresis by chromatography on a Sephadex G-25 column equilibrated in a 1:5 dilution of electrophoresis buffer, and the fractions eluting at the void volume of the column were combined and lyophilized. The residue was dissolved in 20% of its original volume in H_2O, adjusted to 1% NaDodSO_4, 50 mM dithiothreitol, and 10% sucrose, and tracking dye was added. After heating for 30 min at 37 °C, an aliquot of the sample was then applied to a 0.6×10 cm polyacrylamide gel that had been electrophoresed for 2 h prior to sample application. The gels were then electrophoresed for 4 h at 8 mA/gel. [^{3}H]Proline-containing collagenous proteins secreted by CHL cells were prepared and electrophoresed in an identical fashion.

For radioactively labeled samples, the completed gels were sliced into 2 mm segments using a Gilson gel fractionator, and the samples were dried overnight at 37 °C. The residue was then digested with 0.2 mL of 30% H_2O_2 for 1.5 h at 60 °C, and each sample was suspended in 10 mL of Aquasol after cooling. Radioactivity in each segment was then measured in a Beckman refrigerated liquid scintillation counter. Recovery of applied radioactivity was greater than 85%, and the counting efficiency of the isotope was approximately 30%.

Molecular weight markers for gel calibration were myosin (mol wt ~210 000), cross-linked γ-globulin heavy chains (mol wt ~100 000), bovine serum albumin (mol wt ~67 000), aldolase (mol wt 50 000), and γ-globulin light chains (mol wt ~25 000). The relative mobility of the standards was visualized after staining with 0.05% Coomassie Brilliant Blue, and their position of migration is depicted at the top of Figure 2.

Results

The HT1 clone of CHL cells synthesizes and secretes into the growth medium substantial quantities of collagen-like

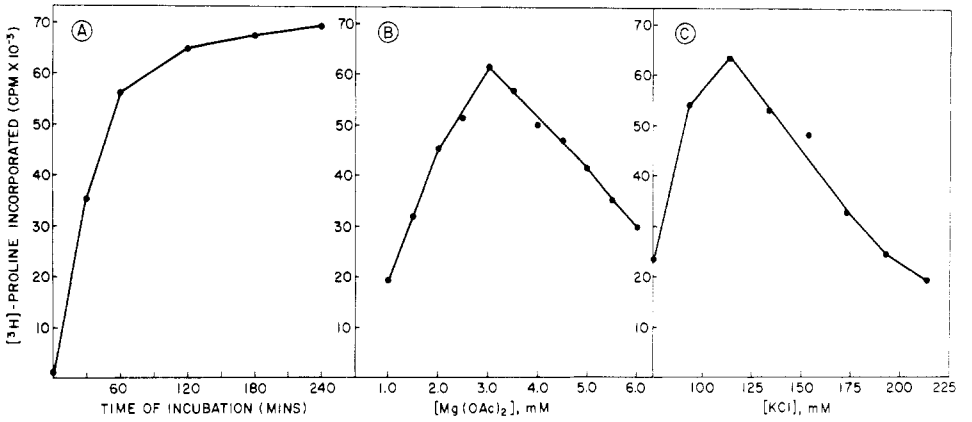

FIGURE 1: Reaction conditions for [²H]proline incorporation catalyzed by CHL cell-free preparations. Standard protein synthesis reactions supplemented with 0.72 A_{260} unit of CHL S30 were incubated at 25 °C for the indicated time (A) or for 2 h at various $Mg(OAc)_2$ (B) or KCl (C) concentrations as indicated. Values reported were corrected for nonspecific binding of radioactivity to the filter in the absence of extract (580 cpm).

TABLE I: Reaction Requirements for Cell-Free Protein Biosynthesis Catalyzed by Chinese Hamster Lung Cell Extracts.[a]

	Polypeptide synthesized			
	[³H]Proline		[³H]Leucine	
Reaction components	cpm	pmol	cpm	pmol
Complete	61 200	1.61	74 400	1.54
Minus extract	430	0.01		
Minus incubation	600	0.02		
Minus ATP, GTP, CPK, and CP	9 600	0.25		
Plus puromycin (0.67 mM)	4 800	0.13		

[a] Cell-free incorporation of ³H-labeled amino acids into protein was performed as described under Experimental Procedure with modification of standard reaction mixtures as indicated. Reaction mixtures were supplemented with 0.62 A_{260} units of CHL S30 and were incubated for 2 h at 25 °C. Values presented were corrected for radioactivity retained by the filter in the absence of extract (0.01 and 0.02 pmol for [³H]proline and [³H]leucine, respectively).

TABLE II: Collagenase Effects on CHL S30 Catalyzed Reaction Products.[a]

	Acid-insoluble [³H]proline (cpm)		
Component	Minus collagenase	Plus collagenase	% collagenase digestible product
A. CHL S30 amount (A_{260} units)			
0.18	22 700	12 200	46.3
0.37	37 800	19 200	49.2
0.75	55 100	29 100	47.2
B. CHL S30 + collagen	59 200	60 600	0

[a] Standard reaction mixtures, containing CHL S30 extract (A) in the amounts indicated or (B) 0.75 A_{260} unit, were incubated for 2 h at 25 °C. The mixtures were then adjusted to 2 mM in respect to $CaCl_2$, supplemented with collagenase when indicated, and incubated an additional 90 min at 37 °C, and acid-insoluble [³H]proline labeled products were determined as described in Experimental Procedures. In B, 100 μg of rat skin collagen was added to the reaction mixture before the addition of collagenase. Values have been corrected for nonspecific binding of [³H]proline to the filters in the absence of extract (745 cpm).

material, and studies are currently in progress to define and quantitate the precise biochemical nature of this material. This report describes the conditions under which CHL cell-free extracts catalyze the biosynthesis of collagen-like products. As illustrated in Table I, dialyzed cell-free extracts prepared from CHL cells promote the incorporation of approximately the same amount of [³H]leucine or [³H]proline in a highly efficient manner (~2.5 pmol of amino acid/A_{260} unit of extract) into newly synthesized protein, a result consistent with the observation that the cells produce and secrete collagen. The observation that the relatively nonabundant amino acid, proline, was incorporated into newly synthesized material to the same extent as a common amino acid, leucine, suggested that collagen-like protein(s) could represent one of the major cell-free synthesized products.

Preceding product characterization, conditions affecting total protein synthesis catalyzed by the cell-free extract were evaluated. As shown in Table I, the CHL S30-catalyzed incorporation of [³H]proline into newly synthesized material is dependent upon the extract and incubation, is stimulated approximately sixfold by the additon of nucleoside triphosphates and a regenerating system, and is inhibited greater than 90% by the addition of the antibiotic, puromycin. Figure 1A illustrates that the incorporation of radioactive substrate into acid-insoluble material is linear with respect to time for 1 h at 25 °C followed by a slower rate of synthesis which continues for several hours. The reaction has $Mg(OAc)_2$ and KCl optima at 3 and 113 mM, respectively (Figures 1B and 1C). These reaction parameters are the same if [³H]leucine is used in place of proline (data not shown). We have also observed that addition of spermine to the reaction mixture does not affect the amount of proline incorporated or alter the $Mg(OAc)_2$ and KCl optima.

In order to determine if the cell-free synthesized material contained collagenous products, the completed in vitro reaction mixtures were subjected to digestion with bacterial collagenase. The enzyme preparation used in this laboratory has been treated with Tos-LysCH_2Cl to inactivate clostripain (Porter et al., 1971), and under the incubation conditions used for these experiments (90 min at 37 °C in the presence of reducing agent) this material contained no detectable proteolytic activity against noncollagen proteins (aldolase, γ-globulin, bovine serum albumin, or ovalbumin) as determined by either fluorescamine assay (Böhlen et al., 1973) or polyacrylamide gel electrophoresis (data not shown). When completed reaction mixtures were incubated with this enzyme preparation, approximately 50% of the incorporated [³H]proline was rendered acid soluble (Table II, part A). Furthermore, the addition of

100 μg of rat skin collagen, to the cell-free synthesized products before the exposure to collagenase, prevented the solubilization of the cell-free incorporated radioactivity (Table II, part B). The observations that a constant proportion of the cell-free synthesized material was digested regardless of the amount of total product exposed to the enzyme (Table II, part A) and that the presence of nonradioactive substrate for the enzyme protected the radioactive products of the reaction against degradation by collagenase (Table II, part B) serve as additional evidence for the specificity of the collagenase used in these experiments. Furthermore, we have determined that treatment of the newly synthesized material with collagenase causes a decrease from 45 400 to 24 800 cpm in the amount of acid-insoluble radioactivity eluting at the void region of a G-25 column (data not shown). This result indicates that the decrease in acid-insoluble material after collagenase treatment is due to the digestion of the cell-free synthesized material to small molecular weight products and is in quantitative agreement with the filter assay (Table II, part A) in that approximately one-half of the [^{3}H]proline incorporated is contained in collagenase specific sequences.

Preliminary characterization of the cell-free synthesized collagenous proteins was achieved by $NaDodSO_4$-polyacrylamide gel electrophoresis. The gel system used was selected on the basis that other investigators (Park et al., 1975) have detected high molecular weight collagenous proteins under these conditions. As shown in Figure 2A, three distinct regions of radioactivity that are digestible with bacterial collagenase (Figure 2B) are produced by the cell-free system. The material migrating with a relative mobility of 165 000 daltons comprises 20% of the total product and based upon size and enzyme sensitivity is thought to represent cell-free synthesized complete procollagen α chains. Two additional peaks of collagenase sensitive radioactive material are also observed: (1) a species with a mol wt of 95 000 comprising 13% of the material; and (2) material with a mol wt of approximately 65 000 representing 12% of the product formed. Thus, 45% of the material produced by the extract can be correlated with three molecular weight species each being susceptible to collagenase digestion. As shown in Figure 2C, cultured CHL cells secrete into the medium three species of collagenase sensitive (Figure 2D) proteins that have molecular weights (175 000, 98 000, and 60 000) which approximate those of the cell-free synthesized products (165 000, 95 000, and 65 000). The appearance in the medium of a high molecular weight collagenase sensitive species not observed in the products of the cell-free system is presently thought to reflect the absence of β-aminopropionitrile in the medium which allowed covalent cross-linking of α chains to occur (Tanzer, 1973). Nevertheless, the data presented indicate that the cell-free system catalyzes the synthesis of collagenous proteins that appear to correlate in size with products secreted by the cells. Molecular weight analysis of the cell-free synthesized material further confirms the observation that a significant fraction (45%) of the product formed is collagenous in nature, with 20% of the total corresponding in size to procollagen α chains.

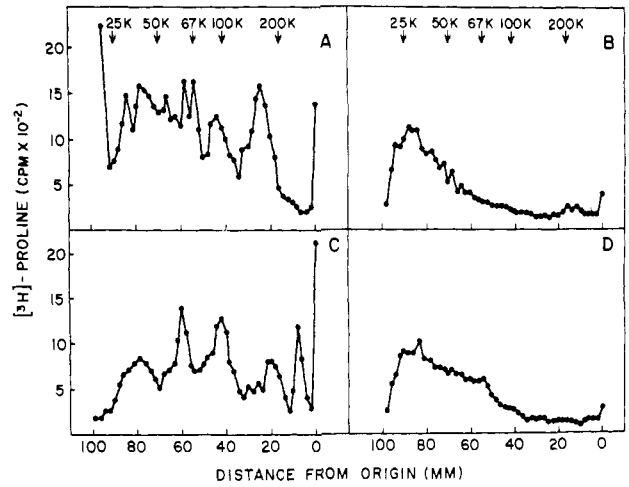

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell-free synthesized or secreted collagenous proteins. Samples were prepared and electrophoresed, and radioactivity was measured as described in Experimental Procedures: (A) cell-free synthesized reaction products, 48 350 cpm; (B) cell-free synthesized reaction products after collagenase digestion (27 400 cpm); (C) [^{3}H]proline labeled proteins secreted by CHL cells into medium (34 000 cpm); (D) [^{3}H]proline-labeled secreted proteins after collagenase digestion (20 334 cpm). The relative mobilities of molecular weight standards are indicated at the top of the figures. Direction of electrophoresis is from right to left.

Discussion

Cell-free extracts prepared from the HT1 clone of Chinese hamster lung cells catalyze the biosynthesis of collagenous material in a highly efficient manner with 20% of the product corresponding in size to procollagen α chains. In this system, the cell-free synthesis of high molecular weight material occurs under conditions of optimal total protein synthesis. Elevated KCl levels are not required, as reported by others (Benveniste et al., 1976; Harwood et al., 1975) for the formation of large products. A second collagenase digestible species representing 13% of the total product made corresponds in size to processed α chains. Other investigators (Kerwar et al., 1973; Vuust & Piez, 1972) have reported the processing of proα chains to α chains presumably due to the action of procollagen peptidase or nonspecific protease activity present in other S30 fractions. Additionally, material of similar size represents the major collagenous product detected when other mammalian protein synthesis systems are used (Lazarides & Lukens, 1971; Benveniste et al., 1973; Wang et al., 1975). Thus, approximately one-third of the total product made in this cell-free system is considered to be initially synthesized as the proα species. A third species (mol wt 65 000) that is at least partially sensitive to collagenase is also produced by the cell-free reaction. Although a collagenase sensitive material corresponding in size to this product is secreted by CHL cells (Figure 2C), the origin and nature of this material are unknown. It may arise from proteolysis that appears to have occurred in the reaction, from translation of a different mRNA species, or it may be the product directed by partially degraded mRNA. Work is now in progress to resolve this question as well as to determine the type of proα chain collagen produced by CHL cells in culture and by the cell-free extracts.

Although our data are consistent with the currently favored concept that each chain in procollagen is translated from three separate mRNAs rather than being a single chain (Church et al., 1971), the apparent processing of proα chains in our system does not allow a definitive answer to this question. Final resolution of the multiple chain vs. single chain models of procollagen must await translation of a purified procollagen mRNA in a translation system such as wheat germ in which collagen is not a natural product and which theoretically should be free of specific procollagen processing proteases. We currently are isolating mRNA from CHL cells in order to ascertain the nature of the initially translated product. Chinese hamster lung cells possess several characteristics in culture which have facilitated their use in a variety of both biochemical and genetic studies (Haralson & Roufa, 1975; Roufa & Reed, 1975). These same properties—a rapid generation time (ca.

14 h at 37 °C), a stable karyotype, and growth to high cell density under a variety of culture conditions—also make this cell line suitable as a source of relatively large amounts of mRNA. In this laboratory, we routinely grow 10 g of CHL cells containing 500 A_{260} units of polysomes from which approximately 1 mg of mRNA can be extracted (Haralson & Roufa, 1975; Haralson & Mitchell, unpublished observations). Since 20–30% of the total cell-free product is procollagen material (data from this paper), it is felt that several hundred micrograms of procollagen mRNA can be routinely obtained using this source. Thus, CHL cells should provide a suitable source of sufficient quantities of procollagen mRNA to allow definitive characterization of the initially synthesized product using cell-free protein synthesis methodologies.

A further advantage offered by CHL cells is the ability of cell-free extracts derived from this source to catalyze the biosynthesis of collagenous material in vitro. In view of recent reports (Carpousis et al., 1977; Collins & Crystall, 1975) that collagen synthesis may be regulated at the translational level, such a system provides a unique approach to dissecting and describing those elements that regulate the synthesis. Since methodologies for isolating active protein synthesis components from CHL cells are established (Haralson & Roufa, 1975), it should be possible by using procollagen mRNA derived from these cells in combination with both homologous CHL or heterologous wheat germ and rabbit reticulocyte translational systems to elucidate those factors which affect collagen mRNA translation. Thus, CHL cells offer a unique practical system for determining not only the structure of initially synthesized procollagen but also the regulation of its synthesis.

References

Bańkowski, E., & Mitchell, W. M. (1973) *Biophys. Chem. 1,* 73–86.

Beneveniste, K., Wilczek, J., & Stern, R. (1973) *Nature (London) 246,* 303–305.

Benveniste, K., Wilczek, J., Ruggieri, A., & Stern, R. (1976) *Biochemistry 15,* 830–835.

Boedtker, H., Crkvenjakov, R. D., Last, J. A., & Doty, P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4208–4212.

Boedtker, H., Frischauf, A. M., & Lehrach, H. (1976) *Biochemistry 15,* 4765–4770.

Böhlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155,* 213–220.

Carpousis, A., Christner, P., & Rosenbloom, J. (1977) *J. Biol. Chem. 252,* 2447–2449.

Chiu, J. F., & Sung, S. C. (1970) *Biochim. Biophys. Acta 209,* 34–42.

Church, R. L., Pfeiffer, S. E., & Tanzer, M. L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 2638–2642.

Collins, J. F., & Crystal, R. G. (1975) *J. Biol. Chem. 250,* 7332–7342.

Davidson, J. M., McEneany, L. S. G., & Bornstein, P. (1975) *Biochemistry 14,* 5188–5194.

Diaz deLeon, L., Paglia, L., Breitkreutz, D., & Stern, R. (1977) *Biochem. Biophys. Res. Commun. 77,* 11–19.

Fairbanks, G. Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10,* 2606–2617.

Fessler, L. I., Morris, N. P., & Fessler, J. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4905–4909.

Haralson, M. A., & Roufa, D. J. (1975) *J. Biol. Chem. 250,* 8618–8623.

Harwood, R., Connolly, A. D., Grant, M. E., & Jackson, D. S. (1974) *FEBS Lett. 41,* 85–88.

Harwood, R., Grant, M. D., & Jackson, D. S. (1975) *FEBS Lett. 57,* 47–50.

Kerwar, S. S. (1974) *Arch. Biochem. Biophys. 163,* 609–613.

Kerwar, S. S., Cardinale, G. J., Kohn, L. D., Spears, C. L., & Stassen, F. L. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 1378–1382.

Lazarides, E., & Lukens, L. N. (1971) *Nature (London), New Biol. 232,* 37–40.

Lee-Own, V., Zeichner, M., Benveniste, K., Denny, P., Paglia, L., & Slavkin, H. C. (1977) *Biochem. Biophys. Res. Commun. 74,* 849–856.

Monson, J. M., Click, E. M., & Bornstein, P. (1975) *Biochemistry 14,* 4088–4092.

Morris, N. P., Fessler, L. I., Weinstock, A., & Fessler, J. H. (1975) *J. Biol. Chem. 250,* 5719–5726.

Murphy, W. H., von derMark, K., McEneany, L. S. G., & Bornstein, P. (1975) *Biochemistry 14,* 3243–3250.

Park, E., Tanzer, M. L., & Church, R. L. (1975) *Biochem. Biophys. Res. Commun. 63,* 1–10.

Pawlowski, P. J., Gillette, M. T., Martinell, J., Lukens, L. N., & Furthmayer, H. (1975) *J. Biol. Chem. 250,* 2135–2142.

Porter, W. H., Cunningham, L. W., & Mitchell, W. M. (1971) *J. Biol. Chem. 246,* 7675–7682.

Prichard, P. M., Staton, G. W., Jr., & Cutroneo, K. R. (1974) *Arch. Biochem. Biophys. 163,* 178–184.

Roufa, D. J., & Reed, S. J. (1975) *Genetics 80,* 549–566.

Tanzer, M. (1973) *Science 180,* 561–566.

Uitto, J., Lichtenstein, J. R., & Bauer, E. A. (1976) *Biochemistry 15,* 4935–4942.

Vuust, J., & Piez, K. A. (1972) *J. Biol. Chem. 247,* 856–862.

Wang, L., Simoes, C. L., Sonohara, S., Brentani, M., Andrade, H. D., Jr., da Silva, S. M. F., Salles, J. M., Marwues, N., & Brentani, R. (1975) *Nucleic Acids Res. 2,* 655–666.